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Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF

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Several members of the tumour necrosis factor receptor (TNF-R) superfamily can induce cell death. For TNF-R1, Fas/APO-1, DR3, DR6, TRAIL-R1 and TRAIL-R2, a conserved 'death domain' in the intracellular region couples these receptors to activation of caspases. However, it is not yet known how TNF receptor family members lacking a death domain, such as TNF-R2, CD40, LT-βR, CD27 or CD30, execute their death-inducing capability. Here we demonstrate in different cellular systems that cytotoxic effects induced by TNF-R2, CD40 and CD30 are mediated by endogenous production of TNF and autotropic or paratropic activation of TNF-R1. In addition, stimulation of TNF-R2 and CD40 synergistically enhances TNF-R1-induced cytotoxicity. These findings describe a novel pro-apoptotic mechanism induced by some members of the TNF-R family.

Keywords: apoptosis/Bcl-2/Bcl-x_L/CrmA/TNF receptor

Introduction

Members of the tumour necrosis factor (TNF) family are important for the functioning of the immune system, tissue homeostasis and embryonic development. At the cellular level, these cytokines activate cell differentiation, cell proliferation, cell survival or cell death, depending on the metabolic state of the cell (Grell and Clauss, 1999). The ability to kill cells has attracted great attention ever since the direct cytotoxic effect of the prototypic ligand of this family, TNF, was discovered (Fiers, 1993; Ashkenazi and Dixit, 1998). Molecules of the TNF ligand family exert their effects through members of the TNF receptor (TNF-R) family. These are characterized by homologies in their extracellular region containing two to six copies of a canonical motif of cysteine-rich pseudorepeats. This family includes, for example, the transmembrane receptors TNF-R1, TNF-R2, Fas/APO-1, CD27, CD30, CD40, LT-βR and TRAIL receptors 1, 2, 3 and 4, as well as a variety of soluble receptors of mammalian and viral origin (Smith *et al*., 1994; Grell and Clauss, 1999).

TNF-R family members show little similarity in their cytoplasmic region with two exceptions. First, several members of the family can bind to a group of homologous proteins termed TRAFs (TNF-R-associated factors). However, there is no clearly defined domain within those receptors to which the TRAF proteins bind with the exception of a PXQXT motif found in CD30, TNF-R2, RANK and CD40 (Arch *et al*., 1998). Secondly, a homologous sequence of ~65–80 amino acids, termed the 'death domain' (DD), was originally identified in Fas/APO-1 and TNF-R1 (Itoh and Nagata, 1993; Tartaglia *et al*., 1993). This domain was sequentially found in other members of the TNF receptor family that can induce cell death, namely DR3, DR6, TRAIL-R1 and TRAIL-R2 (Ashkenazi and Dixit, 1998). In the case of TNF-R1 and Fas/APO-1 it has been shown that induction of apoptosis involves DD oligomerization (Boldin *et al*., 1995) and rapid (within seconds) activation of caspases (Kischkel *et al*., 1995; Boldin *et al*., 1996). Caspases are cysteine proteases which cleave certain proteins after specific aspartic acid residues (Thornberry and Lazebnik, 1998). Recruitment and activation of caspase-8 to stimulated 'death receptors' is mediated by DD-containing adaptor proteins such as TRADD and FADD/MORT1 (Ashkenazi and Dixit, 1998).

Surprisingly, it was found that certain members of the TNF receptor family, which lack a DD, are also capable of inducing cell death under certain conditions. For instance, TNF-R2 was shown to kill certain cells when over-expressed (Heller *et al*., 1992; Vandenabeele *et al*., 1995; Haridas *et al*., 1998) and can trigger apoptosis at physiological expression levels in the rhabdomyosarcoma cell line KYM-1 (Grell *et al*., 1993, 1994). Moreover, an involvement of TNF-R2 in activation-induced cell death (AICD) was deduced from experiments using TNF-R-deficient mice (Zheng *et al*., 1995). The stimulation of CD30, which is expressed on the surface of tumour cells of Hodgkin's disease, anaplastic large cell lymphomas (ALCL) and lymphoid cells (Stein *et al*., 1985), was shown to induce cell death of ALCL-derived cell lines and to enhance T cell receptor ligation-induced apoptosis of T hybridoma cells (Smith *et al*., 1993; Lee *et al*., 1996). In addition, experiments with CD30-deficient mice have indicated that this receptor is involved in the deletion of autoreactive thymocytes (Amakawa *et al*., 1996). CD40 plays a critical role in T cell-dependent B cell activation, but can also transmit a death signal in transformed cells of mesenchymal and epithelial origin (Hess and Engelmann, 1996). Finally, stimulation of the receptor for the hetero-trimeric LT-α/LT-β ligand, LT-βR, can induce

apoptosis in some adenocarcinoma cell lines in the presence of interferon-γ (IFN-γ) (Browning *et al*., 1996).

In contrast to the wealth of information about the molecular mechanisms of apoptosis signaling by DD-containing receptors, little is known about the processes that link DD-lacking receptors of the TNF-R family to the cell death machinery. In the case of CD30, it was shown that the C-terminal 66 amino acids, which bind to TRAF1 and TRAF2, are essential for the ability of CD30 to enhance T cell receptor signaling-induced apoptosis of T hybridoma cells (Lee *et al*., 1996). For LT-βR, ligand-induced association of TRAF3 was implicated in cell death signaling, because a deletion mutant of TRAF3 potently inhibited LT-βR- but not Fas/APO-1-transduced apoptosis (VanArsdale *et al*., 1997). Interestingly, TRAF3 has also been reported to interact with CD30, TNF-R2 and CD40, and has been implicated in CD40-mediated growth inhibition of epithelial cells (Eliopoulos *et al*., 1996). In addition, TRAF-mediated binding of RIP2, a Ser/Thr kinase, to CD40 has been shown recently (McCarthy *et al*., 1998). Overexpression of RIP2 induces both activation of NF-κB transcription factors and apoptosis, but its physiological role in CD40 signaling remains to be determined.

Apart from direct induction of cell death, DD-lacking receptors of the TNF-R family have been shown to enhance the cytotoxic effects of DD-containing receptors in certain conditions (Grell *et al*., 1995; Hess and Engelmann, 1996; Weiss *et al*., 1997, 1998). Although the underlying mechanisms for this phenomenon are not entirely understood, interference of the signaling pathways from DD-containing receptors and DD-lacking receptors at the level of TRAF molecules has been suggested. It has been proposed that this involves DD-lacking receptor-induced sequestration and degradation of TRAF molecules (Duckett and Thompson, 1997; Weiss *et al*., 1998). Here we analyzed the molecular mechanisms by which DD-lacking receptors of the TNF-R family induce cell death and show that triggering of TNF-R2, CD40 and CD30 leads to an upregulation of TNF production and autotropic or paratropic stimulation of TNF-R1-mediated cytotoxicity.

Results

TNF-R1- and TNF-R2-induced apoptosis of KYM-1 cells can both be antagonized by inhibition of caspases but not by Bcl-2 or Bcl-x_L

The rhabdomyosarcoma cell line KYM-1 was previously shown to be highly sensitive for the induction of cell death induced by ligation of either of the two TNF receptors, and thus represents an ideal model to compare the pro-apoptotic signaling pathways of the two receptors (Grell *et al*., 1993). Stimulation of either of the two TNF receptors leads to comparable morphological and biochemical changes in KYM-1 cells, characteristic for apoptotic cell death. Experiments using various signal transduction inhibitors have suggested differences in the activation of the apoptotic machinery (Grell *et al*., 1994). To investigate the role of caspases in the pro-apoptotic signaling pathways activated by TNF-R1 and TNF-R2, we tested the effects of various peptide inhibitors of caspases on TNF-R1- or TNF-R2-induced cell death in KYM-1 cells. The cytotoxic effects of TNF-R1- and TNF-R2-stimulation were both inhibited by different inhibitors in a similar range of concentrations (Figure 1A and B). The broad spectrum caspase inhibitor zVAD-fmk inhibited TNF-R1- and TNF-R2-transduced apoptosis in the nanomolar range. The peptide inhibitors zDEVD-cmk and Ac-YVAD-CHO, which have modest specificity for caspase-3 and caspase-1, respectively (Thornberry *et al*., 1997), affected cell death only at high concentrations (50–100 μM). The cowpox virus-encoded protein CrmA is a potent inhibitor of different pro-inflammatory and pro-apoptotic caspases, such as caspase-1 and caspase-8, and blocks TNF- and FasL-induced cell death *in vitro* and *in vivo* (Strasser *et al*., 1995; Smith *et al*., 1996). Expression of CrmA in KYM-1 cells not only completely blocked cytotoxicity induced by TNF (data not shown) or by antibody mediated stimulation of TNF-R1, but also blocked TNF-R2-induced cell death (Figure 1C–E). These results provide evidence that both TNF receptors use similar members of the caspase family to induce cell death.

For Fas/APO-1 two different apoptosis signaling pathways have been identified (Strasser *et al*., 1995; Scaffidi *et al*., 1998). In one cell type, induction of apoptosis by Fas/APO-1 occurred under essential involvement of mitochondria and could be counteracted by overexpression of Bcl-2 or Bcl-x_L, whereas in another cell type overexpression of Bcl-2 or Bcl-x_L had no effect. Caspase-8 was activated in both cell types although with different kinetics. To analyze which of these pathways are involved in TNF-R1- and TNF-R2-induced apoptosis, we transfected expression constructs for several anti-apoptotic molecules into KYM-1 cells. Overexpression of Bcl-2 or Bcl-x_L did not protect KYM-1 cells significantly against cytotoxicity induced by stimulation of either TNF-R1 or TNF-R2 (Figure 1F–K). Bcl-2 or Bcl-x_L did, however, inhibit cell death induced by staurosporine, etoposide or γ-irradiation, demonstrating that functional levels of these proteins were expressed in these KYM-1 transfectants (data not shown). Taken together, the data indicate that TNF-R2 and TNF-R1 activate apoptosis by a similar mechanism, which requires a CrmA-sensitive caspase, most probably caspase-8, and is insensitive to Bcl-2 and its homologs.

TNF-R2-induced cell killing is mediated by an indirect mechanism via increased production of endogenous TNF and TNF-R1 activation

In previous experiments we had observed that morphological and biochemical changes of apoptosis occur in KYM-1 cells more slowly after TNF-R2 activation compared with TNF-R1 stimulation (Figure 2A; Grell *et al*., 1994). This indicated that TNF-R2 might induce apoptosis by an indirect mechanism. An important hint for the mechanisms of TNF-R2-induced cytotoxicity comes from a recent study showing an involvement of minute amounts of endogenously produced TNF in the cytotoxic effects of huTNF-R2 overexpressed in the mouse fibrosarcoma cell line L929 (Vercammen *et al*., 1995). In fact, we found that TNF-R2-induced cytotoxicity, but not TNF-R1-induced cell death, in KYM-1 cells could be blocked by human TNF-specific neutralizing mAb 357-101-4 (Figure 2B and C), mAb T1 and mAb 195 (data not shown). Even 2 h after TNF-R2 stimulation, addition of antibodies to TNF could still block cytotoxicity

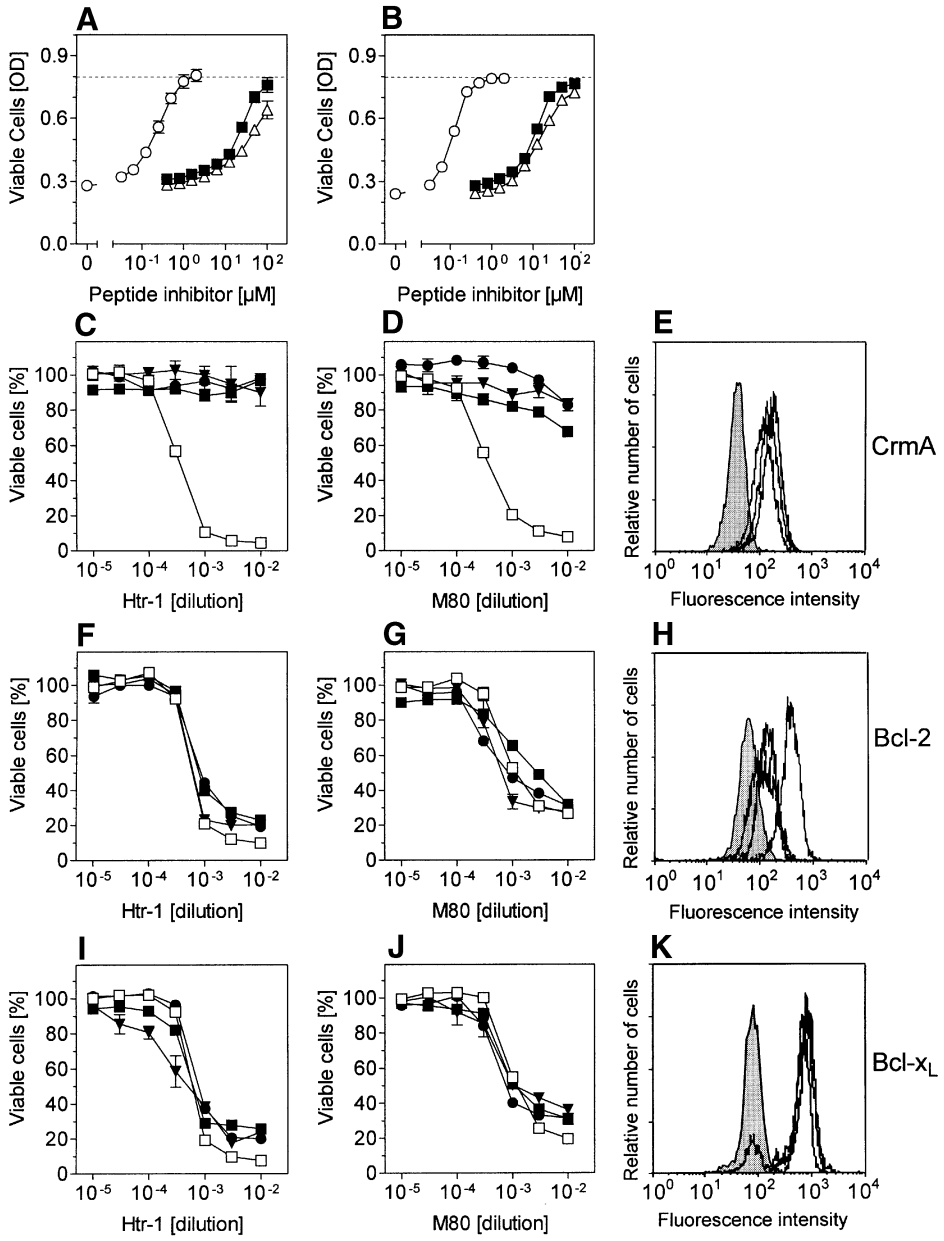

Fig. 1. TNF-R1- and TNF-R2-induced apoptosis in KYM-1 cells are both blocked by inhibition of caspases but not by overexpression of Bcl-2 or Bcl-x_L. (**A** and **B**) KYM-1 cells were pre-treated for 30 min with the indicated concentrations of the peptide inhibitors zVAD-fmk (○), zDEVD-cmk (■) and YVAD-CHO (△) and subsequently treated overnight with (A) the TNF-R1-specific mAb Htr-1 (1:500) or (B) polyclonal TNF-R2-specific rabbit IgG M80 (1: 500). Cell viability was determined by MTT assays and values obtained from untreated cells are indicated by dashed lines. (C–K) KYM-1 clones, stably transfected with expression plasmids for CrmA (**C**–**E**), Bcl-2 (**F**–**H**) or Bcl-x_L (**I**–**K**) were treated overnight with titrated concentrations of the TNF-R1-specific agonistic mAb Htr-1 (C, F and I) or polyclonal TNF-R2-specific rabbit IgG M80 (D, G and J), and cell survival was determined by MTT assays. For each cell death inhibitor results from three independent transfected clones are shown (filled symbols) versus untransfected cells (open symbols). Representative experiments ($n \geqslant 3$) are shown and data points represent mean values of triplicates ± SD. (E, H and K) Expression of the transgene-encoded proteins (solid line) was determined by immunofluorescence staining and FACS analysis. Staining of control vector transfected cells is shown as shaded histograms.

(Figure 2D) consistent with the idea that TNF-R2 triggers cell death indirectly via the induction of endogenous TNF. Furthermore, addition of soluble TNF-R1-Fc chimeric proteins, which neutralize TNF, also blocked TNF-R2-induced cytotoxicity whereas a similar Fas/APO-1-Fc construct had no effect (data not shown). This indicates that induction of endogenous TNF but not FasL is essential for TNF-R2-induced cytotoxicity. RNase protection assays (Figure 2E) and RT–PCR analysis (Figure 2F) revealed that the stimulation of either TNF-R leads to increased expression of TNF mRNA in KYM-1 cells. The time course of TNF-R2-induced upregulation of TNF mRNA (detectable after 2 h) is consistent with the observation that TNF-R2-induced cytotoxicity can be blocked by TNF-specific antibodies as late as 2 h after TNF-R2 stimulation (Figure 2D).

Next we analyzed whether or not TNF induced by TNF-R2 stimulation mediates its cytotoxic effects via TNF-R1 binding. In fact, cell death induced by two distinct TNF-R2-specific stimuli, TNF-R2-specific antibodies or high

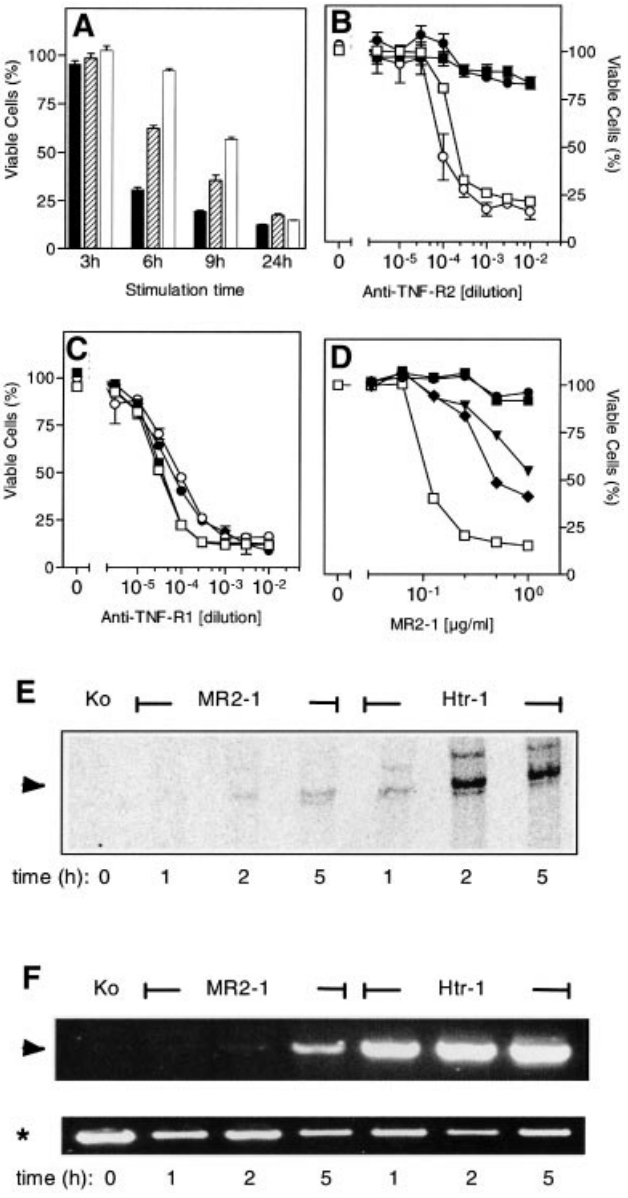

Fig. 2. TNF-R2-induced apoptosis in KYM-1 cells is mediated by endogenous TNF. (**A**) TNF-R2-induced apoptosis is delayed in comparison with TNF-R1-induced apoptosis. KYM-1 cells were treated for the indicated periods with high concentrations of TNF (1 µg/ml; filled bars), TNF-R1-specific mAb Htr-1 (10 µg/ml; hatched bars) and polyclonal TNF-R2-specific rabbit serum M80 (1:30; open bars). Cell viability was determined by MTT assays. (B and C) TNF-R2- but not TNF-R1-induced apoptosis is inhibited by neutralizing anti-TNF antibodies. KYM-1 cells were incubated overnight in the absence (open symbols) or presence (filled symbols) of anti-TNF mAb 357-101-4 (30 µg/ml) with the following reagents: (**B**) polyclonal TNF-R2-specific rabbit IgG M80 (squares) or mAb MR2-1 (circles); (**C**) TNF-R1-specific mAbs Htr-1 (squares) or mAb Htr-9 (circles). (**D**) Time dependence of the neutralization of TNF-R2-induced cell death. KYM-1 cells were treated with titrated concentrations of the TNF-R2-specific mAb MR2-1 and neutralizing anti-TNF mAb 357-101.4 was added after 1 h (●), 2 h (■), 3 h (▼) or 5 h (◆), or was omitted (□). Cell viability in (A–D) was determined by MTT assays. (E and F) Determination of TNF expression. KYM-1 cells were treated for the indicated times with the TNF-R1-specific mAb Htr-1 (2 µg/ml) or TNF-R2-specific mAb MR2-1 (1 µg/ml) and expression of TNF mRNA was examined by RNase protection assay (**E**) or RT–PCR analysis using human TNF-specific primers (**F**). The arrows indicate the TNF-specific nucleic acid fragments and the asterisks indicate β-actin-specific nucleic acid fragments, the level of which was determined as a control for the RT–PCR.

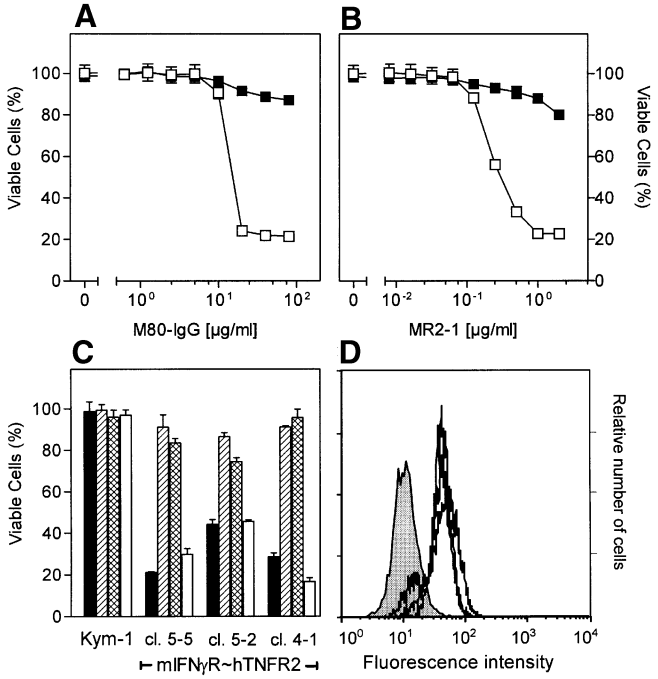

Fig. 3. TNF-R2-induced apoptosis in KYM-1 cells is mediated via TNF-R1. (A and B) KYM-1 cells were cultured overnight with the agonistic TNF-R2-specific reagents polyclonal IgG M80 (**A**) and mAb MR2-1 (**B**), in the absence (□) or presence of neutralizing TNF-R1-specific monovalent (Fab)-fragments of mAb H398 (■). (**C**) KYM-1 cells were stably transfected with a chimeric receptor construct consisting of the extracellular domain of the mouse IFN-γ receptor and the cytoplasmic part of the human TNF-R2 (mIFNγR-hTNFR2). The cells were cultured overnight with mouse IFN-γ alone (10 ng/ml; filled bars) or in the presence of neutralizing anti-TNF mAb 357-101-4 (30 µg/ml; hatched bars), neutralizing (Fab)-fragments of TNF-R1-specific mAb H398 (30 µg/ml; cross-hatched bars), or neutralizing (Fab)-fragments of TNF-R2-specific polyclonal IgG M80 (30 µg/ml; open bars). The results using three independent transfected clones and untransfected KYM-1 cells are shown. (**D**) Expression of the chimeric mIFNγR-hTNF-R2 molecules in three different clones (solid lines) was determined by immunofluorescence staining and FACS analysis. Staining of untransfected cells is shown in the filled histogram.

concentrations of a TNF-R2-specific mutant of TNF ($TNF_{D143N-A145R}$), could be blocked by neutralizing antibodies to TNF-R1 (Figure 3A and B; data not shown). We wanted to exclude the remote possibility that these data resulted from (i) interactions between the extracellular domains of the two TNF receptors (e.g. ligand-induced TNF-R1/TNF-R2 heterocomplexes) or (ii) minimal cross-reactivities of the TNF-R2-specific reagents with TNF-R1. This could be achieved by generating chimeric receptors consisting of the extracellular and the transmembrane domain of mouse IFN-γ receptor and the cytoplasmic part of human TNF-R2. The extracellular part of the mouse IFN-γ receptor was chosen because mouse IFN-γ does not bind to human IFN-γ receptors on KYM-1 cells. Transfection of this chimeric receptor construct into KYM-1 cells yielded clones (Figure 3D) which could be killed by mouse IFN-γ whereas control KYM-1 clones were resistant (Figure 3C). Strikingly, the cytotoxic effects of mouse IFN-γ on these transfectants could be blocked by neutralizing TNF- or TNF-R1-specific antibodies but not by TNF-R2-specific antibodies (Figure 3C).

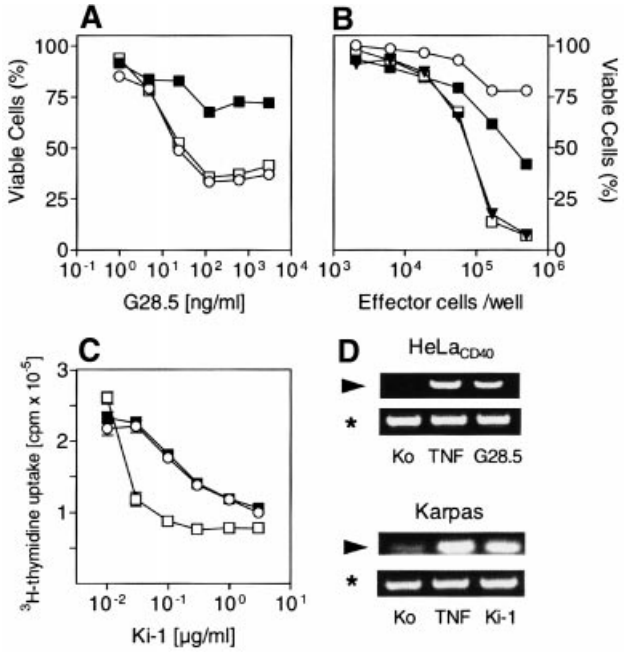

Fig. 4. A role for endogenous TNF in CD40- and CD30-induced cytotoxicity. (**A**) Human HeLa-CD40 cells were treated overnight with the concentrations indicated of the CD40-specific mAb G28.5 alone (□), in the presence of neutralizing anti-human TNF mAb (30 µg/ml, ■), or isotype-matched control mAbs (○). Cell viability was determined by neutral red uptake. (**B**) Mouse A9-CD40 cells were co-cultured with paraformaldehyde-fixed wild-type BHK cells (○) or BHK cells expressing human CD40L in the absence (□) or presence of neutralizing anti-mouse TNF mAb MP6-XT22 (■), or, as a control, neutralizing anti-human TNF mAb T1 (▼). (**C**) Human Karpas-299 lymphoma cells were treated overnight with titrated concentrations of the CD30-specific mAb Ki-1 in the absence (□) or presence of neutralizing TNF-specific mAb 195 (■) or TNF-R1-specific (Fab)-fragments of mAb H398 (○). Cell proliferation was determined by [^{3}H]thymidine uptake. (**D**) The cells indicated were treated for 5 h with TNF (10 ng/ml), the CD40-specific mAb G28.5 (1 µg/ml), the CD30-specific mAb Ki-1 as indicated or left untreated and expression of TNF mRNA was examined by RT–PCR using human TNF-specific primers. The arrows indicate the TNF-specific nucleic acid fragments and the asterisks indicate β-actin-specific nucleic acid fragments.

CD40- and CD30-induced cell death is also mediated via increased production of endogenous TNF and TNF-R1 activation

Next, we investigated whether the cytotoxic effects of other DD-lacking receptors of the TNF-R family might be transduced indirectly via production of endogenous TNF. Ligation of CD40 has been shown to result in apoptotic cell death in cells of mesenchymal and epithelial origin (Hess and Engelmann, 1996). In HeLa cells stably transfected with CD40 (HeLa-CD40), induction of cytotoxicity by an agonistic CD40-specific antibody could be antagonized by neutralizing anti-human TNF mAbs (Figure 4A). Moreover, in the mouse cell line A9, transfected with CD40 (A9-CD40), cytotoxicity induced by co-culture with CD40L-transfected BHK cells could be inhibited, albeit not completely, by mouse TNF-specific antibodies (Figure 4B). Determination of TNF mRNA by RT–PCR analysis revealed that stimulation with anti-CD40 antibodies or, as a control with TNF, induced TNF mRNA expression in HeLa-CD40 cells (Figure 4D).

CD30-induced cytotoxicity in the human Karpas-299 lymphoma cell line (Smith *et al*., 1993) could also be partially inhibited by TNF- or TNF-R1-specific antibodies (Figure 4C). In addition, stimulation of Karpas-299 cells with CD30-specific mAbs or TNF led to an increase in TNF mRNA expression (Figure 4D). The inhibition of CD30-induced cytotoxicity by neutralizing TNF- or TNF-R1-specific antibodies was not as potent as in the case of TNF-R2- and CD40-induced cell death. This indicates that CD30-stimulation can activate cell death by several mechanisms, only one of which involves production of TNF. FasL does not appear to be involved since we found no inhibition of CD30-induced cell death by addition of Fas/APO-1-Fc (data not shown). The extent by which anti-TNF antibodies inhibited CD30-induced cell death varied considerably between individual experiments. The reasons for this variable involvement of endogenous TNF in CD30-induced cytotoxicity are not known, but might be caused by the development of variable TNF resistance by low level spontaneous TNF expression in these cells (Figure 4D).

TNF-R2-, CD40- and CD30-stimulation induce cell death through activation of TNF-R1 by membrane-anchored TNF

To determine the amount of TNF produced after TNF-R2 stimulation we analyzed the supernatants of TNF-R2-stimulated KYM-1 cells for soluble TNF protein. Interestingly, using a highly sensitive ELISA, we could not detect significant levels of soluble TNF in the supernatants of KYM-1 cells that had been stimulated via TNF-R2 (Figure 5A, left panel). However, increased levels of TNF were present in the detergent soluble fractions of KYM-1 cells stimulated with TNF-R2-specific agonistic antibodies or KYM-1-muIFNγR-TNF-R2 cells treated with mouse IFN-γ (Figure 5A, right panel). In addition, we found that the supernatant of TNF-R2-stimulated KYM-1 cells did not contain soluble factors which are cytotoxic for human KYM-1 cells or mouse L929 cells (Figure 5B), consistent with the notion that only insignificant amounts of soluble TNF are released by these cells. Similar results were obtained from CD40-stimulated cells (data not shown). Finally, inhibition of the protease which processes the transmembrane form of TNF into soluble trimers by TAPI (Black *et al*., 1997) did not significantly inhibit TNF-R2-induced cytotoxicity in KYM-1 cells (Figure 5C). This confirms that generation of soluble TNF trimers is not an essential step in this process. Collectively, these data demonstrate that stimulation of TNF-R2 and other DD-lacking receptors of the TNF-R family upregulates production of endogenous TNF which in its transmembrane form kills cells via an autotropic or paratropic manner by binding to TNF-R1.

TNF-R2-, CD40- and CD30-stimulation can enhance TNF-R1-induced cell death by a mechanism that is independent of endogenous TNF production

We have recently shown that costimulation of TNF-R2 can strongly enhance TNF-R1-induced cytotoxicity in cellular systems in which cytotoxicity is completely dependent on TNF–TNF-R1 interaction (Grell *et al*., 1995; Weiss *et al*., 1997, 1998). We therefore examined whether this effect is also exerted by an indirect mechanism involving TNF-R2-induced upregulation of TNF production. We investigated the nature of this cooperation under conditions in which stimulation of TNF-R2 alone did not

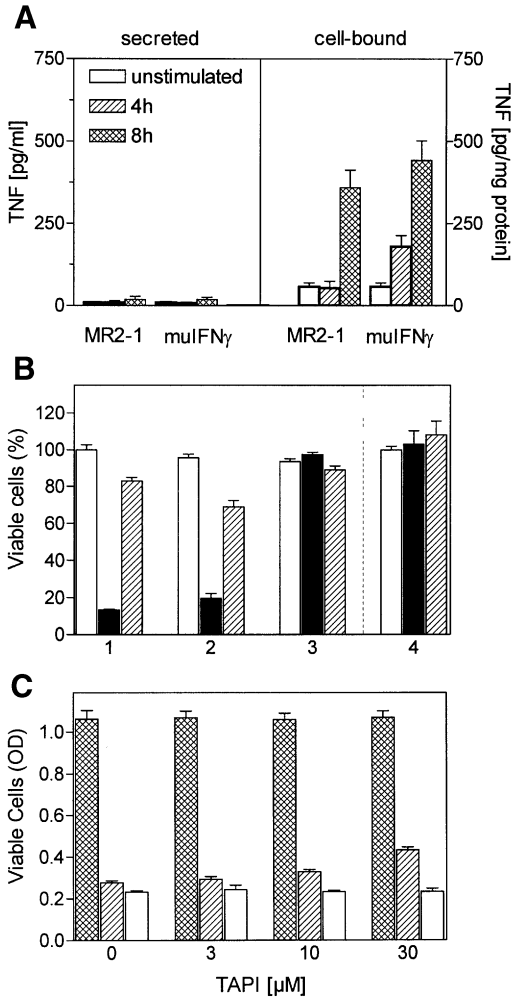

Fig. 5. TNF-R2-induced cytotoxicity in KYM-1 cells is mediated by endogenous membrane-anchored TNF. (**A**) KYM-1 cells were stimulated for the times indicated via the endogenous TNF-R2 using mAb MR2-1 (1 μg/ml) or via chimeric muIFNγR-huTNF-R2 receptors using mouse IFN-γ (10 ng/ml). TNF protein concentrations were determined in the cell supernatants (left panel) or the detergent soluble cell fractions (right panel) by a TNF-specific ELISA. Bars represent mean values ± SEM of three independent experiments each performed in duplicate or triplicate. (**B**) No cytotoxic activity is detectable in the supernatant of KYM-1 cells stimulated with agonistic TNF-R2-specific antibodies after protein A depletion. In (1), KYM-1 cells were treated with TNF-R2-specific polyclonal rabbit IgG M80 in the absence (filled bars) or presence (hatched bars) of anti-human TNF mAb 357-101-4 (30 μg/ml) or left untreated (open bars). In (2), centrifuged supernatant from cells treated as described in (1) were transferred onto fresh KYM-1 cultures. In (3), centrifuged supernatant from cells treated as described in (1) were incubated for 2 h with protein A–Sepharose beads, cleared by centrifugation and transferred onto fresh KYM-1 cultures. In (4), centrifuged supernatant from cells treated as described in (1) were transferred onto cultures of L929 cells and 1 μg/ml actinomycin D was added. Control groups revealed L929 cells to be highly sensitive for soluble human TNF (detection limit 1 pg/ml) but resistant to the huTNFR-2-specific polyclonal rabbit IgG M80 (data not shown). (**C**) KYM-1 cells were stimulated with TNF-R2-specific agonistic mAb MR2-1 (hatched bars), TNF-R1-specific agonistic mAb Htr-1 (open bars) or medium as a control (cross-hatched bars) in the presence of different concentrations of TAPI, an inhibitor of the TNF-converting enzyme. Cell viability in (B) and (C) was determined by MTT assays.

induce significant cytotoxic effects per se. We found that the cytotoxic effects of the agonistic TNF-R1-specific antibody Htr-1 on KYM-1 cells after a short incubation period of 6 h was synergistically enhanced in the presence of agonistic TNF-R2-specific antibodies (Figure 6A). At that time point no effect of the TNF-R2-specific agonistic antibody alone could be observed (see 0 value in Figure 6A). In a second experimental setting, we observed that the cytotoxic effects of an overnight incubation with the agonistic TNF-R1-specific mAb Htr-1 could be strongly enhanced when co-incubated with TNF-R2-specific agonistic antibodies. This was done in the presence of neutralizing anti-TNF antibodies to block endogenous TNF (Figure 6B). Again under these conditions, no significant cytotoxic effect of TNF-R2 stimulation alone could be observed. Stimulation of CD40 on HeLa-CD40 cells also strongly enhanced TNF-R1-induced cytotoxicity, even under conditions in which endogenous TNF was neutralized (Figure 6C). These data confirm earlier findings that stimulation of DD-lacking receptors of the TNF-R family, such as TNF-R2, CD40 and CD30, can enhance TNF-R1-induced cytotoxic effects (Grell *et al*., 1995; Hess and Engelmann, 1996; Duckett and Thompson, 1997) by a mechanism that is independent of production of endogenous TNF. From the data of this study we deduce a novel model for the autoregulatory mechanism by which DD-lacking receptors of the TNF-R family can participate in the induction of cell death by two different, but amplifying mechanisms (Figure 7). Accordingly, the stimulation of TNF-R2, CD40 and CD30 (i) leads to an upregulation of cytotoxic ligands, such as TNF, and (ii) sensitizes to apoptosis induced by DD-receptor activation.

Discussion

The induction of cell death is an essential part of normal life itself. The remarkable processes of tissue remodeling during embryogenesis and the extensive cell turnover in numerous adult tissues, such as hemopoietic cells, skin cells and intestinal cells, illustrate the importance of a regulated mechanism to eliminate cells (Jacobson *et al*., 1997). Cell death control has been adapted to a variety of physiological needs, particularly in complex organisms. Therefore numerous mechanisms for cell death signaling have evolved, many of which are the subject of intensive research. One way for an organism to get rid of unwanted cells is the selective cell contact-mediated lysis of virus-infected target cells by cytotoxic T cells. Other mechanisms involve soluble cytotoxic factors, such as cytokines, steroid hormones or glutamate, to which cells are sensitive only under certain conditions. One fascinating mechanism of cell death signaling is initiated by interaction of members of the TNF ligand family with their cellular receptors (Ashkenazi and Dixit, 1998).

Identification and molecular cloning of a variety of receptor-associated cytoplasmic factors have unraveled how stimulation of DD-containing members of the TNF-R family, ‘death receptors’, can trigger apoptosis (Nagata, 1997; Ashkenazi and Dixit, 1998). The DD of TNF-R1, Fas/APO-1, DR3, DR6, TRAIL-R1 and TRAIL-R2 couple activated receptors to DD-containing adaptor molecules such as TRADD and FADD/MORT1. A second homophilic interaction motif present in these adaptors, the death effector domain (DED), couples the activated signaling complex to the initiator caspase, caspase-8, which directly or indirectly initiates degradation of essential cellular

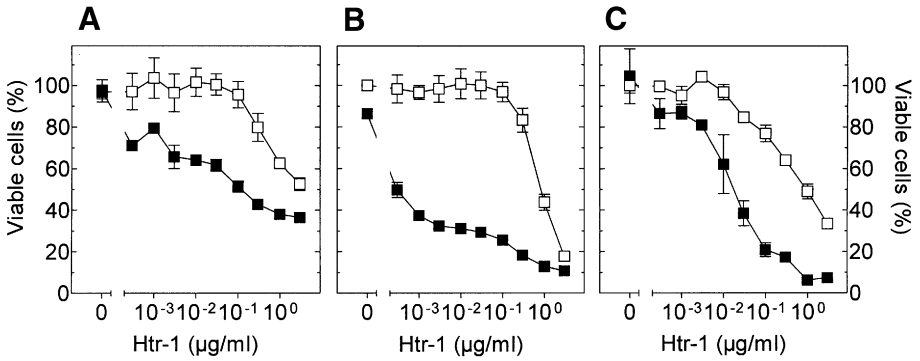

Fig. 6. Stimulation of TNF-R2 and CD40 enhances TNF-R1-induced cytotoxicity independently of endogenous TNF. (**A**) KYM-1 cells were treated with the TNF-R1-specific mAb Htr-1 in the absence (□) or presence (■) of TNF-R2-specific polyclonal rabbit IgG M80 and cell viability was determined after 6 h by MTT assays. (**B**) KYM-1 cells were incubated with anti-human TNF mAb 357-101-4 (30 µg/ml) and then cultured for 18 h with titrated concentrations of TNF-R1-specific mAb Htr-1 in the absence (□) or presence (■) of TNF-R2-specific mAb MR2-1 (0.1 µg/ml). (**C**) HeLa-CD40 cells were incubated with neutralizing anti-human TNF mAb 195 (30 µg/ml) and then cultured for 18 h with CHX (2.5 µg/ml) and titrated concentrations of TNF-R1-specific mAb Htr-1 in the absence (□) or presence (■) of CD40-specific mAb G28.5 (1 µg/ml).

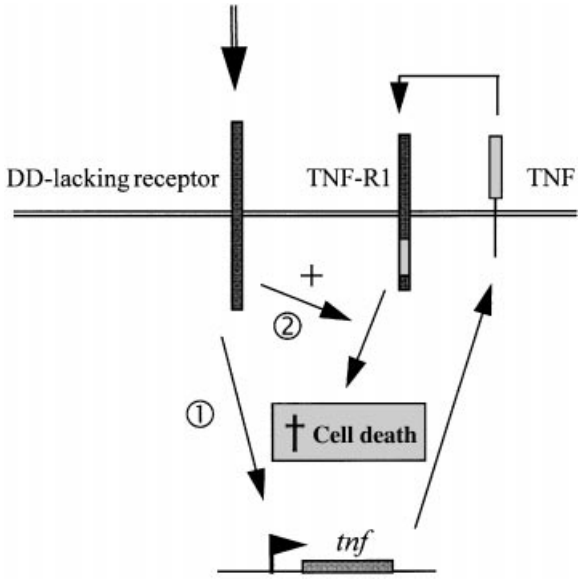

Fig. 7. Model for cell death signaling by stimulation of DD-lacking members of the TNF-R family. See text for details.

proteins. How signals from DD-lacking TNF-R family members are coupled to the cell death machinery has until recently been an open question. Here, we demonstrate in several cellular systems, that induction of cell death by TNF-R2, CD40 and, with some restrictions, CD30 is mediated indirectly through production of endogenous membrane-anchored TNF and signaling via TNF-R1.

At first glance, triggering of cell death via induction of cytotoxic ligands and subsequent DD-receptor activation seems to be an attractive and simple way to explain the cytotoxic effects of various compounds and ligands. TNF-R2-, CD40- and CD30-induced cytotoxicity is somewhat paradoxical, since these receptors are also known to initiate signaling pathways that promote cell survival, cell proliferation and cell differentiation (Grell and Clauss, 1999). CD40-ligation promotes proliferation and differentiation of B lymphocytes and is essential for humoral immune responses, whereas stimulation of TNF-R2 and CD30 enhance T cell activation. The nature of these signaling pathways is not fully understood, but it is assumed that recruitment of members of the TRAF family and other adaptor molecules leads to activation of AP-1 and NF-κB transcription factors and expression of cell growth promoting genes (Arch *et al*., 1998). Interestingly, activation of NF-κB and the JNK/AP-1 signaling pathway appear to be essential for the induction of TNF and FasL expression, at least in activated T lymphocytes (Jongeneel, 1995; Swantek *et al*., 1997; Kasibhatla *et al*., 1998). It is therefore conceivable that signals which initiate cell proliferation and cell activation are accompanied by the production of cytotoxic cytokines, such as TNF or FasL. This mechanism may be involved in self-limitation of immune responses by killing effector cells. It appears, however, that TNF-producing cells, such as activated macrophages or T lymphocytes, are resistent to the cytotoxic effects of TNF, at least during the initial stages of activation.

Consistent with an anti-apoptotic function of NF-κB and AP-1 signaling is the observation that their activation renders cells resistant to TNF-R1 stimulation (Ashkenazi and Dixit, 1998). Why then does induction of TNF expression by stimulation of TNF-R2, CD30 and CD40, all of which also activate NF-κB and AP-1, lead to TNF-R1-transduced apoptosis rather than protect cells from the toxic effects of TNF? A possible explanation for this finding comes from data showing that stimulation of TNF-R2, CD40 and CD30 potently enhances TNF-R1-transduced cell death (Duckett and Thompson, 1997; Weiss *et al*., 1997; this study). Several mechanisms have been suggested by which TNF-R1- and Fas/APO-1-induced apoptosis signaling can be amplified at the level of DD-containing adaptor proteins (Varfolomeev *et al*., 1996). In addition, release of cytochrome *c* and activation of the pro-apoptotic Bcl-2 family member Bid have been proposed as a mechanism for amplifying TNF-R1- and Fas/APO-1-induced caspase activation (Li *et al*., 1998; Luo *et al*., 1998; Scaffidi *et al*., 1998). However, our data showing that overexpression of Bcl-2 or Bcl-x_L in KYM-1 cells does not protect KYM-1 cells against TNF-R1- and TNF-R2-induced cell death (Figure 1) argue against an involvement of such a mechanism. Consistent with this observation, it was shown that although Bcl-2 and Bcl-x_L can antagonize Bid, they do not inhibit TNF-induced cell death (Gross *et al*., 1999). We and others demonstrated previously that enhancement of TNF-R1-

induced cell death by TNF-R2 or CD30 signaling depends on the ability of these latter receptors to recruit TRAF2. We therefore prefer the idea that degradation of TRAF2 or interference with its function is the mechanism by which TNF-R2, CD40 and CD30 stimulation enhance the cytolytic activity of TNF-R1 (Duckett and Thompson, 1997; Weiss *et al*., 1998). According to this hypothesis, prolonged stimulation of TNF-R2, CD40 or CD30 would lead to removal of protective proteins from TNF-R1, rendering the cell more sensitive to TNF-R1-induced cytotoxicity. Interestingly, the finding of the potent TNF-R2-mediated enhancement of TNF-R1-induced cell death (Figures 6 and 7) explains why reagents which significantly but not completely inhibit cell death induction by TNF-R1 fail to block TNF-R2-induced cell death (Grell *et al*., 1994).

Consistent with our observation that the amount of TNF produced upon TNF-R2-, CD30- and CD40-stimulation is rather low (Figure 5), overexpression and stimulation of huTNF-R2 in mouse L929 fibroblasts resulted in partial cytotoxicity, which could be neutralized by mouse TNF-specific antibodies, although TNF protein could not be detected (Vercammen *et al*., 1995). Our discovery that upon TNF-R2-stimulation TNF is upregulated in its membrane-anchored form without significant processing to the soluble trimers (Figure 5) provides an explanation for this phenomenon. It is pertinent that membrane-anchored TNF and FasL are more potent inducers of apoptosis than their soluble trimers, and it is possible that the former is the only one that triggers cell death under physiological conditions (Eissner *et al*., 1995; Grell *et al*., 1995; Schneider *et al*., 1998; Strasser and O'Connor, 1998; Tanaka *et al*., 1998). In line with this argument is recent work demonstrating that ligation of CD40 on hepatocytes can lead to an upregulation of Fas/APO-1 and FasL, and subsequent autocrine/paracrine apoptosis (Afford *et al*., 1999).

Apart from indirect induction of cell death by TNF-R2, CD40 and CD30 via upregulation of TNF and FasL, other more direct effects of these receptors on cell viability must also be considered. This is suggested by work showing that the Fas/APO-1-independent component of AICD of $CD8^+$ T cells *in vitro* is abrogated in cells of TNF-R2-deficient mice but normal in cells of TNF-R1-deficient animals (Zheng *et al*., 1995). Obviously, a TNF-R2-induced TNF–TNF-R1 interaction cannot take place in this scenario. However, this TNF-dependent component of AICD is a slow process when compared with Fas/APO-1-induced apoptosis. Accordingly, the TNF-R2-activated TRAF2 degradation or interference with TRAF-2 function might be a mechanism by which this process occurs. Deletion of $CD8^+$ T cells induced by triggering with supra-optimal antigen concentrations also relies on a TNF–TNF-R2 interaction without an obvious involvement of TNF-R1 (Alexander-Miller *et al*., 1998). This indicates that TNF-R2 might play a specialized role in the induction of cell death in this subset of T cells. The role of the two TNF receptors in the deletion of peripheral T lymphocytes in the whole animal is still a matter of debate (Sarin *et al*., 1995; Zheng *et al*., 1995; Speiser *et al*., 1996; Zhou *et al*., 1996), and it is clear that distinct pathways for the induction of lymphocyte apoptosis exist (Strasser, 1995).

In conclusion, our study reveals a novel autoregulatory mechanism by which DD-lacking receptors of the TNF-R family, such as TNF-R2, CD40 and CD30 participate in the induction of cell death namely by (i) upregulation of cytotoxic ligands, such as TNF, and (ii) sensitization of cells to apoptosis by a signaling pathway which is independent of endogenous TNF. These data add to the knowledge of how members of the TNF-R family are part of the intricate network of cell death signaling.

Materials and methods

Cells and reagents

The human rhabdomyosarcoma cell line KYM-1 was generously supplied by M.Sekiguchi (University of Tokyo, Japan) and maintained in Click-RPMI medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS). All other cells were grown in RPMI 1640 medium (Biochrom), containing 5% FCS. The human cervical carcinoma cell line HeLa and the mouse fibrosarcoma cell line L929 were obtained from American Type Culture Collection (Rockville, MD). Karpas-299 lymphoma cells have been described previously (Fischer *et al*., 1988). CD40L-transfected BHK cells, CD40-transfected HeLa cells and CD40-transfected A9 cells, a murine L cell derivative, have all been described previously (Hess and Engelmann, 1996). Recombinant human TNF (specific activity 2×10^7 U/mg) was kindly provided by I.-M.von Broen (Knoll AG, Ludwigshafen, Germany). The TNF-R2-specific TNF mutein ($TNF_{D143N\text{-}A145R}$) was kindly provided by H.Loetscher, Basel, Switzerland. Peptide inhibitors of caspases, zVAD-fmk, zDEVD-cmk and YVAD-CHO, were purchased from Bachem AG (Bubendorf, Switzerland). The generation and specificity of the monoclonal antibodies H398 (Thoma *et al*., 1990), Htr-1 and Htr-9 (Brockhaus *et al*., 1990), G28-5 (Clark and Ledbetter, 1986), Ki-1 (Schwab *et al*., 1982), 357-101-4 (Grell *et al*., 1995), 195 (Eissner *et al*., 1995) and 80M2 (Grell *et al*., 1993) have all been described previously. The polyclonal rabbit anti-human TNF-R2-specific IgG M80 and generation of neutralizing monovalent (Fab)-fragments thereof have been described previously (Grell *et al*., 1993). The anti-human TNF monoclonal antibody T1 was generated in our laboratory by standard hybridoma techniques. The human TNF-R2-specific agonistic monoclonal antibody MR2-1 was obtained from HyCult Biotechnology, Uden, The Netherlands. Expression constructs coding for TNF-R1-Fc and Fas/APO-1-Fc chimeric proteins were kindly provided by C.Ware, San Diego, CA. Hybridoma cells producing the mouse IFN-γ receptor-specific rat monoclonal antibody GR20 were obtained from ATCC and the antibody was purified on protein G–Sepharose (Pharmacia) in our laboratory (A.S.). Mouse IFN-γ was purchased from R&D Systems (Minneapolis, MN). The FITC-labeled goat anti-mouse IgG + IgM antibodies were obtained from Dianova (Hamburg, Germany). FITC-labeled goat anti-rat IgG antibodies and all other reagents if not stated otherwise were obtained from Sigma (Deisenhofen, Germany).

Expression constructs and transfections

The expression vectors for Bcl-2 (pEF Bcl-2/neo), FLAG epitope-tagged human Bcl-2 (pEF FLAG-Bcl-2/puro), FLAG-Bcl-x_L (pEF FLAG-Bcl-x_L/puro) and FLAG-CrmA (pEF FLAG-crmA/puro) have been described previously (Strasser *et al*., 1995; Huang *et al*., 1997). The pCDM8/γR-p80 plasmid was generated by replacing the extracellular and transmembrane domains of a pCDM8-based TNF-R2-expression plasmid (Weiss *et al*., 1997) by an amplicon comprising amino acids 1–276 of the mouse IFN-γ receptor using the unique *Bsp*HI site located two amino-acids downstream of the transmembrane domain of TNF-R2 and the *Hin*dIII site of the pCDM8 stuffer. The cDNA for this chimeric receptor was subsequently amplified using the following oligo-nucleotides: 5′-CAAGATCTCGGAATGGGCCCGCAG-3′ and 5′-CGTCTAGATTAA-CTGGGCTTCATCCCAGC-3′, digested with *Bgl*II and *Xba*I, and the resulting fragment subcloned into the *Bam*HI and *Xba*I sites of the pEF-pGK/puro vector (Huang *et al*., 1997) to produce the pEF-muIFNgR-huTNFR2/puro expression vector. The construct was fully sequenced (ABI PRISM™, Perkin-Elmer, Foster City, CA) before transfection of the DNA. Transfections of KYM-1 cells were performed by electroporation using a Gene-pulsar (Bio-Rad, München, Germany) essentially as described (Strasser *et al*., 1995), or by liposome-mediated gene transfer using pFx™-2 (Invitrogen, San Diego, CA) or SuperFect™ (Qiagen, Hilden, Germany), all according to the manufacturers' protocols. Independent clones of transfected cells were picked after selection with

400 µg/ml G418 (Gibco-BRL, Eggenstein, Germany) or 3 µg/ml puromycin (Sigma, Deisenhofen, Germany), and transgene-expressing cells were identified by immunofluorescence staining and flow cytometric analysis on a FACScan (Becton Dickinson, San Jose, CA). Cells were either surface stained or stained after fixation with 1% paraformaldehyde and permeabilization with 0.3% saponin (Sigma) as described previously (Strasser *et al*., 1995), using the primary mAbs mouse anti-human Bcl-2 (Bcl-2-100), mouse anti-FLAG M2 monoclonal antibody (Sigma) or rat anti-mouse IFN-γ-R monoclonal antibody GR20 followed by FITC-labeled secondary antibodies.

RNA analysis

For RNase protection assays, $5–10\times10^6$ cells were treated with the reagents indicated and RNA was subsequently isolated with the RNeasy RNA purification Kit (Qiagen) according to the manufacturer's recommendations. The RNA samples were analyzed for the presence of huTNF transcripts with the RiboQuant RNase Protection Assay System (PharMingen, Hamburg, Germany) according to the manufacturer's recommendations using the Multi-Probe human cytokine template set hCK-3. L32 and GAPDH were included as internal controls. Protected transcripts were separated by polyacrylamide gel electrophoresis under denaturing conditions and signals were quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For RT–PCR analyses, total RNA of stimulated cells was isolated and transcribed into cDNA using the First-Strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations. TNF-specific PCRs were performed using the following primers: forward, 5′-CTT-CTGCCTGCTGCACTTTGGA-3′; reverse, 5′-TCCCAAAGTAGACC-TGCCCAGA-3′, giving a product of 548 bp. PCR cycle conditions were 94°C for 80 s, 60°C for 150 s, 72°C for 180 s, for 30 cycles. Samples were resolved on agarose gels and PCR products visualized with ethidium bromide staining.

ELISA

Cells were seeded in 6-well plates (0.7×10^6 per well) overnight. The next day, the culture medium was changed and 1 h later cells were stimulated in a total volume of 2 ml medium as indicated. The supernatants were kept, the cells were harvested and cell pellets were lysed in 110 µl of 20 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors for 30 min on ice. The lysed cells were centrifuged at 14 000 *g* to remove cellular debris and and samples were calibrated for protein concentrations using the Bio-Rad Protein Assay Kit (Bio-Rad, München, Germany). TNF protein levels in the cellular supernatants and detergent soluble cellular fractions were determined using a human TNF-specific ELISA (PharMingen) according to the manufacturer's protocol, using titrations of human TNF as standard to calibrate the system. Color development was measured at 405 nm with a R5000 ELISA plate reader (Dynatech, Guernsey, GB). Background reactions were determined by omitting the primary ELISA antibody and resulting values were subtracted.

Viability assays

Cell survival assays were performed as described previously (Grell *et al*., 1993, 1995; Hess and Engelmann, 1996). Briefly, KYM-1 cells, HeLa cells, or Karpas-299 cells ($1–3\times10^4$ per well) were seeded into 96-well microtiter plates in a final volume of 200 µl in the presence of the different cell death-inducing reagents. After the indicated times, the metabolic activity or viability of the cells was determined by the MTT-method, by neutral red uptake or [^{3}H]thymidine incorporation as indicated. Cytotoxicity assays using HeLa-CD40 cells were performed in the presence of cycloheximide as described previously (Hess and Engelmann, 1996).

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